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(57) Abstract

A method for preventing the expression of undesired gene products, and a process for improving the method of obtaining desired products from the expression of growing cellular microorganisms, both comprising inserting an altered plasmid, are disclosed and claimed. In particular disclosed are: a method for preventing the expression of the autolytic enzyme (the N-acetylmuramoyl-L-alanine amidase) of *Streptococcus pneumoniae*; a method for introducing the inactivated autolysin gene into any one of the *Streptococcus pneumoniae* strains with chemically different capsules; and a process for using such nonautolysing *pneumococci* for the production of capsular polysaccharides uncontaminated by cell wall material and thus representing a safer and less costly antipneumococcal vaccine.

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AUTOLYSIS-RESISTANT STRAINS AND METHODS EMPLOYING SAME

This invention relates to autolysis resistant pneumococcal strains and methods for making and employing such strains. More particularly, this invention relates to pneumococcal strains having the gene which produces autolysin interrupted by an inserted plasmid so that the enzyme is not produced and organisms comprising said strains do not self-destruct; and, this invention relates to processes for making and employing such strains. More generally, this invention relates to a method for preventing the expression of undesired gene products during the growth of a cellular microorganism without substantially affecting the other aspects of its phenotype which comprises insertional inactivation of the appropriate gene(s) in the microorganism, and growing the microorganism in an appropriate medium. Likewise, this invention relates to improvements in a method for obtaining desired gene products from a growing cell by inhibiting the expression of undesired gene products through insertional inactivation of the appropriate gene(s) in the cell and growing the cell in an appropriate medium.

BACKGROUND OF THE INVENTION

DNA cloning and methods for constructing recombinant DNA techniques are known, see, e.g., Goodenough, U., Genetics, Ch. 14, especially secs. 14.5 to 14.9, pp. 547 to 552 (Saunders College, Philidephia/Holt Rinehart and Winston 1978), incorporated herein by reference.

In a typical protocol for the construction of recombinant DNA, a plasmid or phage cloning vehicle or vector is selected which can be cut by a restriction enzyme such as EcoR1 without losing its ability to self-replicate. Examples of cloning vehicles include Col E1, pSC101. The cloning vehicle is linearized by the restriction enzyme; and, this "linearized plasmid" is next treated with an exonuclease which attacks and digests the 5′ ends of duplex DNA to create single-stranded "tails" at the 3′ ends. Examples of such exonucleases include those derived from phage λ. Tracts of poly-adenine (poly-A) are then added to these tails. This addition reaction can be mediated by an enzyme terminal transferase. The DNA to be cloned, i.e., the plasmid or recombinant plasmid is similarly treated with exonuclease and terminal transferase: the terminal transferases add tracts of poly-thymine (poly-T) to the 3′ ends of the recombinant plasmid. The preparations of the cloning vehicle and the recombinant plasmid are admixed: the poly-A tracts anneal with the poly-T tracts to form a circular structures; and, in a final step, are covalently sealed with ligase.

Pneumococci have remained major human pathogens in spite of the introduction of antibacterial agents such as penicillin into chemotherapy. There is general agreement that while the rate of mortality from pneumococcal disease has declined, the attack rate has remained unchanged and morbidity and even mortality form this bacterial disease plateaued off at unacceptably high levels (e.g., 30% for meningitis), in spite of the availability and general use of antibiotics. The

importance of finding more effective interventions against pneumococcal disease has gained emphasis by the emergence and worldwide spread of penicillin-resistant and multi-resistant penumococcal strains and by the shift in the patient populations (in the Western world) in the direction of a higher proportion of the aged who are known to be particularly susceptible to pneumococcal disease. Thus, intervention of pneumococcal infection through vaccination has remained an important, even if so far unfulfilled, public health goal all over the world.

Various microorganisms are employed in the production of compounds which are either useful as or are useful to prepare products such as vaccines. For instance, Streptococus pneumoniae is used in the production of antigenic polysaccharides to prepare vaccines against S. pneumoniae infection. In general, microorganisms which express or which are caused to express (e.g., by recombinant DNA techniques) a gene for a useful product are grown to maximal cell density. For example, with respect to S. pneumoniae, maximal cell density may be achieved by growing the microorganisms into the stationary phase of growth. The desired product produced during the growth phase is thereafter separated from the microorganisms.

More particularly, pathogenic strains of S. pneumoniae belong to any one of a large number (83) of different capsular types, most of which act as distinct antigens in a human host. For instance, antibodies produced against a strain expressing capsular polysaccharide type

3 will not react with another strain that expresses capsular polysaccharide type 2 on its surface. For this reason, all effective antipneumococcal vaccines are multi-component vaccines, composed of mixtures of chemically distinct capsular polysaccharides. For instance, one of the vaccines includes polysaccharides from 23 different strains which represent over 95% of pathogenic pneumoccocci. This vaccine is marketed as Pneumovax^{R}23 (Merck).

In the procedures used for the preparation of the polysaccharide material, the producer cultures are allowed to grow to maximal cell density, e.g., into the stationary phase of growth, in order to have optimal yield of material. At about the end of the exponential phase of growth, the separation of the capsular polysaccharides is usually conducted to make them available for the production of vaccines. To obtain the polysaccharides, the first step is the separation of the producing bacteria from the culture medium since the latter contains most of the capsular polysaccharides in soluble form. The broth is then further fractionated for the isolation of the bacterial polysaccharides.

A problem which has been encountered is that microorganisms carry a gene for the production of autolysin. In pneumococci, this gene is the lytA gene, which, when expressed, causes the microorganism to produce the enzyme called autolysin. After the culture of the microorganism has reached a maximal cell concentration (stationary phase) the autolysin becomes activated causing destruction of the cell wall, i.e.,

the organism lyses and dies. Autolysis releases toxic (inflammatory) materials (e.g., cell wall materials) into the culture medium thereby contaminating polysaccharide vaccine preparations. The products of autolysis are inflammatory and can cause adverse reactions such as erythema and induration at injection site, fever, rashes, hives, vomiting, diarrhea, and even, in some cases, anaphylactoid reactions.

In particular, polysaccharide preparations are contaminated by cell wall material which is released from the bacteria due to the triggering of the activity of the major pneumococcal autolytic enzyme (the N-acetylmuramoyl-L-alanine amidase, to be referred to as "amidase") in the stationary phase of growth. This contamination is responsible for adverse reactions from vaccines; and, cell wall material derived from pneumococci is highly inflammatory as shown in several models of pneumococcal disease.

For instance, with respect to Pneumovax^{R}23, the Physician's Desk Reference (41st ed., 1987) advises that in a study of Pneumovax^{R}22 (22 capsular types) 71% of adults tested had local reactions, e.g., soreness and/or induration at the injection site; and, that rash, arthralgia, fever and even anaphylactoid reactions have been reported. With respect to other capsular polysaccharide vaccines at least 51% of the individually tested experienced adverse reactions. These adverse reactions are a result of contaminants in the vaccines, particularly cell wall material in the vaccines.

The origin of the contaminating wall material in the vaccine preparations is the process of autolysis: the major agent of which is a cell wall degrading enzyme, a so-called autolysin. As more fully discussed herein, this enzyme is ubiquitous in all natural strains of pneumococci and its destructive activity may be triggered by a number of physiologically adverse conditions, such as, e.g., halt in growth in the stationary phase of cultures. Autolysin is also triggered in pneumococci at the end of the exponential growth phase. This latter onset of autolysis coincides with the very time when separation of the capsular polysaccharides is conducted for the production of vaccines. Thus, in the production of vaccines, the culture medium contains soluble cell wall material released by the triggering of autolysin. Further, many of the chemical properties of the desired polysaccharides and of the cell wall materials are sufficiently similar such that removal of the latter through purification techniques is difficult, costly, and not sufficiently effective.

It is thus desired to have autolysis-resistant strains of producer cultures. The lytA gene has been isolated, and a plasmid has been inserted into S. pneumoniae which has resistance to erythromycin as a marker and which interrupts the gene for producing lysin. See Tomasz, A. et al, "Insertional Inactivation of the Major Autolysin Gene of Streptococcus pneumoniae," 170 J. Bacteriology 5931-5934 (Dec. 1988), incorporated herein by reference. However, Tomasz et al do not teach or suggest any methods of employing this plasmid, for example, for

improving the production of useful products from microorganisms by extending the useful life of said microorganisms by preventing them from autolysing, prematurely dying, and contaminating the broth. Furthermore, it is believed that heretofore, no methods employing an altered plasmid for inhibiting the expression of undesired gene products have been taught or suggested.

To overcome the problem of contamination by wall impurities by using genetically altered strains in which the activity of the autolytic amidase is silenced by the inactivation of its structural determinant, the lytA gene, three conditions must be met. First, since natural strains of pneumococci exist in 83 chemically different types (with at least 23 of these types responsible for over 90% of human pneumococcal disease), the genetically altered strains (with inactivated lytA) must include substantially all relevant producer strains, i.e., the method of rendering the lytA gene inactive must be applicable to substantially all relevant producer strains. Secondly, the pneumonococcal strains with the genetically inactivated lytA marker must be fully viable and able to grow at substantially the same rate and in the same media used with lytA active strains. Thirdly, genetically modified strains (with inactivated lytA) must be capable of producing capsular polysaccharide in an immunologically active form. And the capsular polysaccharides from the inactivated LytA strains must be capable of producing vaccines which provide little or no adverse reactions

(especially in comparison to the 51-71% rate of adverse reactions experienced from presently available vaccines).

It is therefore an object of this invention to provide a method employing an altered plasmid which inhibits the production of autolysin and which meets the aforementioned conditions.

SUMMARY OF INVENTION

This invention provides strains, especially pneumococcal strains, having a plasmid inserted therein which results in the gene producing lysin or autolysin, especially the lytA gene, being interrupted so that the enzyme is not produced and organisms comprising said strains do not self-destruct. This invention also provides processes for making and employing such strains. More generally, this invention provides a method for preventing the expression of undesired gene products during the growth of a cellular microorganism without substantially effecting the phenotype which comprises inserting an altered plasmid in the microorganism, and growing the microorganism in an appropriate medium. Likewise, this invention provides an improvement in a method for obtaining desired gene products from a growing cell especially polysaccharide(s) from pneumococcal strains by inhibiting the expression of undesired gene products, especially autolysin, also known as the autolytic enzyme N-acetylmuramoyl-L-alanine amidase, comprising inserting a plasmid in the cell and growing the cell in an

appropriate medium. The plasmid preferably interrupts the lytA coding sequence, and more preferably carries the marker of resistance to erythromycin.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 illustrates the growth rate of pneumonococcal with insertionally inactivated autolysin.

Fig. 2 depicts daughter cell separation at the end of cell division in pneumococcal Lyt- with insertionally inactivated autolysin.

DETAILED DESCRIPTION

Mutants of Streptococcus pneumoniae defective in the major murein hydrolase activity (N-acetylmuramoyl-L-alanine amidase, or amidase) do not undergo autolysis under experimental conditions that cause lysis and cell wall degredation in parental and wild-type cells, but do grow normally, show no apparent defect in genetic transformation and exhibit only a limited degree of chain formation. See Garcia, P., et al, 1986, "Mutants of Streptococcus pneumoniae that contain a temperature sensitive autolysin," J. Gen. Microbiol., 132: 1401-1405; Holtje, J. V., and A. Tomasz, 1976, "Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical homogeneity," J. Biol. Chem. 251:4199-4207; Howard, L. V., and H. Gooder, 1974, "Specificity of the autolysin of Streptococcus (Diplococcus) pneumoniae," J. Bacteriol. 117: 796-804; Lacks, S., 1970, "Mutants of Diplococococcus pneumoniae that lack deoxyribonucleases

and other activities possibly pertinent to genetic transformation," J. Bacteriol., 101:373-381; Mosser, J. L., and A. Tomasz, 1970, "Choline containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme," J. Biol. Chem., 245:287-298; Tomasz A., and S. Waks, 1975, "Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors or cell wall synthesis.," Proc. Natl. Acad. Sci. USA 72: 4162-4166; each of which being hereby incorporated herein by reference.

It has been found that complete and selective suppression of the activity of the major pneumococcal autolytic amidase by insertional activation of the lytA gene does not alter physiological properties and does not substantially change the phenotype, except as to the inhibition of autolysis. Insertional inactivation of the lytA gene is brought about by interrupting the lytA coding sequence by the insertion of a plasmid or a gene, particularly ermC, a staphylococcal gene conferring resistance to erythromycin; and, this resistance to erythromycin is useful as a marker.

Further, it has now been surprisingly found that the expression of undesired gene products can be inhibited or prevented by the insertion of an altered plasmid into a cellular microorganism and then growing the same in an appropriate nutrient medium. Moreover, it has now been surprisingly discovered that the method for obtaining desired products from the gene expression of cellular microorganisms in a culture broth and recovering the

said desired products from the medium or broth and/or the microorganisms is improved significantly by employing microorganisms into which a plasmid has been inserted for preventing expression of undesired products. Preferably, the plasmid comprises an altered plasmid having a detectable marker. More preferably, the desired products comprise pneumonococcal polysaccharides (which are used to prepare vaccines against S. pneumoniae infection), the undesired gene products comprise autolysin or lysin, the altered plasmid comprises an interrupted lytA coding sequence, and the marker is resistance to erythromycin.

In view of this preference, this invention will be further described in terms of S. pneumoniae; but, this is not to be construed as a limitation of the broad concept of this invention. Note that throughout this specification the terms "lysin" and "autolysin" are employed to refer to N-acetylmuramoyl-L-alanine amidase which is also called "amidase".

The following non-limiting examples are given by way of illustration only and are not to be considered a limitation of this invention, many apparent variations of which are possible without departing from the spirit or scope thereof.

EXAMPLES

EXAMPLE 1: Determination of presence and expression of lytA gene in all relevant producer strains of S. pneumoniae.

The structural determinant of autolysin or the amidase, i.e., the lytA gene, was isolated and cloned in E. coli using the procedure described in the second paragraph hereof under Background of the Invention and the DNA representing the lytA gene cloned in the vector plasmid was radiolabeled (^{32}p) using routine procedures for the preparation of DNA probe. This probe was then used to determine whether or not the strains set forth in Tables A, B and C below carry the lytA gene. In particular, the strains of Tables A, B and C were cultured on blood agar plates to form separate colonies. Using routine procedures, each colony was then grown to a liquid culture, lysed and the DNA transferred to membrane filters and probed with the ^{32}p-labeled lytA DNA probe and autoradiography. If the lytA gene was present in the DNA of a strain, the radiolabeled probe would be hybridized (annealed) to the complementary DNA on the membrane filters, resulting in a positive signal upon autoradiography. All of the pneumococcal strains tested positively for the lytA gene. The strains in Tables A, B and C include the relevant vaccine producer strains.

The same strains of Tables A, B and C were cultured on blood agar plates to form separate colonies. Each colony was subjected to a 200 to 500 ug/ml of deoxycholate and/or 10 to 50 ug/ml of the wall hydrolyzing enzyme M1-muramidase. All strains underwent rapid lysis as a result of being exposed to deoxycholate and/or M1-muremidase. Rapid lysis induced by

deoxycholate is a phenomenon uniquely pneumococcal which requires the activity of the pneumococcal autolytic amidase.

Thus, all relevant producer strains of S. pneumoniae carry the lytA gene and express it as well.

Example 2: Production of insertionally inactiviated Lyt$^-$ mutants and properties thereof.

Plasmid pGL80 carrying the lytA gene (See Garcia et al, FEMS Microbiola Lett. 29:77-81 (1985), incorporated herein by reference) was cut with TapI and ligated to the 2.0-kilobase MspI fragment of pE194 containing the ermC gene (See Horinouchi et al, J. Bacteriol., 150:804-814 (1982), incorporated herein by reference). The ligation mixture (DNA, 50 ug/ml) was used to transform S. pneumoniae Rx1 or R6x. Of 102 Em^r transformants, 97 proved to be Lyt$^-$, as judged by resistance to lysis with deoxycholate or by the blue spot assay or both (See Garcia et al, supra). After growth without selection for erythromycin resistance for 50 generations, three of six transformants analyzed showed a stable Em^{r}Lyt$^-$ phenotype; one of these, RUP1, was used for characterizing the mutation. No amidase activity was detectable in crude extracts of RUP1 by using the standard enzymatic assay (See Holtje et al, supra). The Lyt$^-$ phenotype could be transferred linked to Em^r in transformation; when RUP1 DNA was used to transform Rx1, all the Em^r transformants analyzed showed a stable Em^{r}Lyt$^-$ phenotype.

Properties of insertionally inactivated Lyt$^-$ mutants:

(i) Growth rates and adaptive responses

Cultures of the insertionally inactivated Lyt$^-$ mutants and their isogenic Lyt$^+$ parents grew with indistinguishable growth rates to comparable maximal cell concentrations (1 x 10^9 to 5 x 10^9 CFU/ml in stationary phase) at either 37^o or 30^{o}C (doubling times 60 and 140 min, respectively) in a chemically defined medium as described in Tomasz et al, Proc. Natl. Acad. Sci. USA 68:1848-1852 (1971), incorporated herein by reference.

The rate of adjustment to steady-state doubling times was tested in the following situations: a shift from 30 to 37^{o}C and vice versa; a shift from poor to rich medium; and a shift from the stationary to the exponential phase of growth (back dilution). No differences were observable between the strains. On the other hand, the deletion mutant M31 showed a distinctly slower growth rate in the chemically defined medium (doubling times, 85 to 90 min) Fig. 1 depicts the results of this testing by showing the growth rate of the pneumococcal mutant with insertionally inactivated autolysin.

In Fig. 1 cultures of the pneumococcal Lyt$^-$ mutant RUP24 (lyta:ermC) and its isogenic Lyt$^+$ parent R6x were grown in the aforementioned chemically defined medium (A) or in casein hydrolysate medium suplemented with

yeast extract (See Lacks et al, Biochim. Biophys. Acta 39:508-17 (1980), incorporated herein by reference) (B) at 37°C. Cultures of the Lyt⁻ deletion mutant M31 (lytA) were also grown under the same conditions. In the experiments illustrated in panel B, growth temperature was shifted from 37 to 30°C at the time indicated by the arrow. Growth was monitored as optical density (OD) by a spectrophotometer (Sequoia-Turner Spectrophotometer, Mountainview, Calif.). The deletion mutant M31 grew with distinctly slower growth rates, particularly in the chemically defined medium, and cultures of this mutant also showed a longer delay in resumption of exponential growth upon back dilution of a stationary-phase culture into fresh medium. These and other abnormalities of M31 may be related to some function(s) deleted with the DNA in addition to the lytA gene.

(ii) Cell separation at end of cell division

No significant chain formation was observed in Lyt⁺ - Lyt⁻ pairs growing in liquid culture in early or late phase or in young colonies picked from the surface of blood agar plates. Figure 2, and Table 1, below, show daughter cell separation at the end of cell division in the pneumococcal Lyt⁻ mutant with insertionally inactivated autolysin. For the results shown in Fig. 2 and Table 1, cultures of the Lyt⁻ RUP24 and its Lyt⁺ parent R6x were grown in C medium supplemented with yeast extract (Lacks et al, supra). Samples were removed and examined for degree of chain formation in the early log phase (about 1 x 10 CFU/ml) and at the

beginning of the stationary phase of growth (about 1 x 10^9 CFU/ml). Randomly picked fields were scanned by phase-contrast microscopy (Zeiss Research Microscope). Visual observation was preferred to the use of a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), since passage of cells through the orifice of the instrument may cause artifacts (e.g., breaking up of chains). Occasionally, bacteria were first fixed with glutaraldehyde and osmium tetroxide by the previously published procedure (Tomasz et al, J. Cell. Biol. 22:453-467 (1967), incorporated herein by reference). The same isogenic pair of pneumococci was also grown on the surface of tryptic soy agar blood plates, and young colonies (12 < of growth) were picked and scanned for chain formation. Bacteria that appeared to be single cells, doublets, or cells forming chains with three, four, or more than four members were registered, and their frequencies were expressed as the percentage of total cells counted.

TABLE 1

Cell Separation at End of Cell Division

	Pre-Stationary Phase Liquid Cultures		Early Log Liquid Cultures	
	LyT^+	LyT^-	Lyt^+	LyT^-
Total Counted	1336	1516	726	593
Singles	---	---	4.2%	7.2%
Doublets	---	---	90	83
Singles & Doublets	94%	96%	---	---
Threes	0.9	0.5	---	---
Fours	1.2	0.6	---	---
Three & Fours	---	---	0.8	1.8
>Fours	3.3	1.1	3.5	7.2

Young Colonies From Blood Agar Surface	
LyT^+	LyT^-
351	235
---	---
---	---
88%	68%
1.1	11.0
3.7	5
---	---
6.8	14

(iii) Production of Hemolysin

Lyt^- inseritonal mutants plated on the surface of blood agar produced normal alpha-hemolytic zones around the colonies indistinguishable from those surrounding colonies of Lyt^+ cells.

(iv) Genetic transformation

Lyt^+ - Lyt^- pairs of cultures were grown according to a standard procedure used for the induction of competence (See Pozzi et al, Eur. J. Epidemiol. 2:90-94 (1986), incorporated herein be reference), and DNA from strain DP1002 carrying the nov-1 marker was used for transformation (See Holtje et supra). No differences could be observed in the rates of acquisition or levels of competence. At 120 min after dilution of cultures into the competence medium, the Lyt^+ (Rx1) culture had 5.6×10^7 viable cells and 2×10^6 novobiocin-resistant transformants per ml. In the case of the Lyt^- (RUP1) culture, the corresponding numbers were 5.4×10^7 and 1.8×10^6.

(v) Autolysis

Cultures of insertionally inactivated Lyt^- mutants grown at 37°C did not undergo lysis in the stationary phase of growth or when treated with deoxycholate or penicillin (10 x MIC). The same conditions induced some lysis when the Lyt^- cultures were incubating at 30°C.

(vi) Virulence of Lyt^+ - Lyt^- Pair

Isogenic (Lyt^+ - Lyt^-) strains were constructed from encapsulated pneumococci in the following manner: The Lyt^- (Em^r) marker from RUP1 was introduced by genetic transformation into a type 6 clinical isolate (strain A112). The capacity to produce type 3 capsule was transformed into the isogenic pair of R6x (Lyt^+)-RUP24 (Lyt^- Em^r) cells by using transforming DNA isolated from strain HB 565 as the donor of type 3 capsular determinant(s) (Table 2). No significant differences could be detected between the Lyt^+ - Lyt^- pairs in degree of virulence as determined by intraperitoneal injection into mice (Table 3). CD-1 female mice (8 weeks old) were challenged intra-peritoneally with 0.4 ml of bacterial inoculum grown in tryptic soy broth supplemented with glucose (2 mg/ml) and yeast extract (0.1 mg/ml) (See Porter et al, J. Virol. 19:659-667 (1976), incorporated herein by reference) inthe exponential phase of growth. In order to correct for loss of virulence during in vitro growth of penumococci, bacterial strains were first passaged in three consecutive steps in mice in the following manner. Groups of three mice were challenged with large inocula (10^7 to 10^8) of a given strain and killed 24 hour later. The spleens were aseptically removed, homogenized, suspended in tryptic soy broth supplemented with glucose and yeast extract,and incubated at 37°C for 16 to 18 hour. Such a culture was then used to inoculate a second set of animals. After passage 3, groups of 10 mice were challenged with a series of inocula from each

strain and survival rates of mice were monitored by daily observation. The results of this challenge are set forth in Table 3.

TABLE 2

Strains of S. pneumoniae

Strains	Relevant properties*
Recipients in transformation	
Rx1	Cps^- lytA
R6x	Cps^- lytA
A112	cps6A lytA
Donors in transformation	
HB565	cps-3 str-565
DP1002	nov-1
Lyt^- mutants	
RUP1	(Rx1) Cps^- lytA:ermC
RUP24	(R6x) Cps^- lytA:ermC
M31	(R6x) Cps^- lytA
Capsulated isogenic pairs	
RUP25	(R6x) cps-3 lytA:ermC
RUP26	(R6x) cps-3 lytA
RUP20	(A112) cps6A lytA:ermC
RUP21	(A112) cps6A lytA nov-1

* Cps^- Mutation in the genetic determinant(s) for production of capsular polysaccarides; lytA, gene encoding for the major pneumococcal autolysin (amidase); cps-3 and cps6A, genetic determinant(s) for the production of type 3 and type 6A capsular polysaccarides, respectively; lytA:ermC, insertion of ermC into lytA, conferring a stable Lyt^- phenotype (see example, below); nov-1 and str-565, chromosomal point mutations conferring resistance to novobiocin and streptomycin, respectively. (Rx1), (R6x), and (A112) indicate the genetic background of strains obtained by transformation.

TABLE 3

Virulence of Lyt^+ and Lyt^- isogenic pairs

Isogenic pair	No. of dead mice/total no. of mice tested with an inoculum size (CFU) of:		
	10^4	10^5	10^6
RUP20 cps6A Lyt^-	1/10	5/10	10/10
RUP21 cps6A Lyt^+	0/10	8/10	8/10
RUP25 cps3 Lyt^-	10/10	10/10	10/10
RUP26 cps3 Lyt^+	4/10	8/10	10/10

EXAMPLE 3: Vaccines From Lyt^+ - Lyt^- Pair

Isogenic (Lyt^+ - Lyt^-) strains are constructed as set forth in Example 2(vi). Cultures of RUP20 cps6A Lyt^-, RUP 21 cps 6A Lyt^+, RUP25 cps3 Lyt^- and RUP26 cps3 Lyt^+ are grown in a liquid nutrient medium or culture broth. Maximal cell density is achieved in all cultures and at the end of the exponential growth phase, the producer strains are removed from the culture media. The resulting culture supernatants contain, inter alia, the capsular polysaccharides. The capsular polysaccharides are isolated from the culture supernatants by membrane filtration and precipation with hexadecyltrimethylammonium bromide and then purified by physiochemical techniques (see Anderson et al, "Isolation of the capsular polysaccharide from the culture supernatant of Haemophilus influenzae b," Infect. Immun. 1977; 15:472-477, incorporated herein by reference). With respect to polysaccharides from Lyt^+ strains, the products are especially purified from bacterial protein, nucleic acid, endotoxin and cell wall materials since the Lyt^+ strains experience autolysis while the Lyt^- strains do not and the culture supernatants from the Lyt^+ strains contain, inter alia, cell wall materials. The resultant purified polysaccharides are then dissolved in a lactose solution, sterilized by filtration and lyophilized. Sodium chloride is added for isotonicity.

To four groups of thirty mice the Lyt^+ - Lyt^- vaccines are administered subcutaneously: 1 ml (0.15 ug polysaccharide/ml) of vaccine to each mouse. That is, a

first group of thirty mice is given cps6A (Lyt^-) vaccine; a second group is given cps6A (Lyt^+) vaccine; a third group is given cps3 (Lyt^-) vaccine; and, a fourth group is given cps3 (Lyt^+) vaccine. Three weeks after vaccination, these mice are challenged with series of inocula from strains they are vaccinated against, in accordance with the procedure of Example 2(vi) such that of each group of thirty mice ten are given inoculum of size 10^4 CFU, ten are given inoculum of size 10^5 CFU, and ten are given inoculum of size 10^6 CFU, and, in each group of ten, 5 mice are given the Lyt^- strain and 5 are given the Lyt^+ strain, e.g., ten of thirty mice given cps6A (Lyt^-) vaccine are challenged with inoculum of size 10^4 CFU, five of which are challenged with RUP20 cps6A Lyt^- and five of which are challenged with RUP21 cps6A Lyt^+. All of the mice survived; antibodies are produced from the administration of the vaccines.

However, no side effects are observed in the mice given vaccines from Lyt^- cultures. Over fifty percent (35/60) of the mice given vaccines from Lyt^+ cultures experience at least one of the following adverse reactions: erythema and induration at injection site; fever. The adverse reactions generally subside within 48 hours after injection. While the observed side effects from the Lyt^+ vaccines may be considered minor, it is to be noted that in different individuals, more severe adverse reactions are possible.

The above-Examples demonstrate that the present invention meets the conditions stated in the Background of the Invention.

In the preparation of polysaccharide vaccines in accordance with this invention, it is suggested to include lactose, e.g., at a concentration of 2.5 mg/0.5 ml to improve stability (see Tiesjema et al, "Enhanced Stability of meningococcal polysaccharide vaccines by using lactose as menstruum for lyophilization," Bull. World Health Org. 1977; 56:43-48, incorporated herein by reference). Phenol can also be added as a preservative (e.g., 0.25%). Typical doses to achieve an antibody response in humans older than 18 months of age (preferably 24 months or older) is 0.15 to 50 ug polysaccharide(s) per ml with a typical injection having a volume of 0.5 ml. Doses to be administered can be accordingly adjusted by the skilled artisan taking into account such factors as the age, weight, and condition of the patient being vaccinated. Antibodies induced by polysaccharide vaccines may persist for as long as 42 to 48 months or longer after vaccination.

The use of Lyt^- constructs as described and examplified herein, in methods for obtaining products from microorganisms is a significant advancement. For instance, autolysis releases contaminating, toxic and inflammatory materials into the nutrient medium or culture broth used in obtaining products from microorganisms. Autolysis increases the production steps required to make such products by causing the need for purification; autolysis thereby increases the cost of such products. By avoiding autolysis in such methods, safer and less costly products result.

For instance, by avoiding autolysis of producer strain of pneumococcal polysaccharide vaccine preparations, i.e., by employing Lyt$^-$ strains in the process of making the vaccines, safer vaccines and cost savings result because autolysis does not occur and contaminating, toxic and inflammatory materials are not released into the nutrient medium or culture broth.

Table A: Strains from USA

Pt. I.D.	year
K113-106	9/5/78
K101-113	11/14/78
K115-53	11/21/78
K127-38	11/28/78
K131-2	11/27/78
K136-2	11/22/78
K91-154	12/18/78
K94-128	12/18/78
K98-160	12/18/78
K135-3	12/13/78
K100-106	1/9/79
K109-101	1/8/79
K118-61	1/4/79
K119-48	1/30/79
K120-50	1/8/79
K147-1	12/12/79
K130-25	1/14/80
K133-31	1/14/80
K153-3	1/22/80
K134-47	6/2/80
K149-23	11/17/80
116-100	1/6/81
K117-94	1/29/81
K130-44	1/26/81
K126-85	2/23/81
K128-84	3/26/81
K147-23	3/31/81
K165-4	3/16/81
K145-25	4/29/81
K152-25	6/1/81
K156-28	6/15/81
K157-22	6/29/81
K144-37	7/29/81
K136-50	10/12/81
K137-45	10/19/81
K150-23	10/15/81
K134-63	11/18/81
K161-15	12/15/81
K160-28	3/16/82
K169-8	3/22/82
K156-48	4/22/82
K153-47	5/17/82
K154-36	5/11/82
K174-5	8/5/82
K163-21	9/29/82
K171-12	9/20/82
K134-73	10/21/82
K136-61	10/22/82
K156-56	10/13/82
K157-40	10/14/82
K162-53	11/24/82
K168-20	11/10/82

Pt. I.D.	Year
K181-3	11/2/82
K174-13	12/29/82
K130-62	1/13/83
K154-45	1/19/83
K170-24	1/17/83
K135-68	2/17/83
K173-16	2/28/83
K137-63	5/10/83
K190-2	5/11/83
K160-40	7/18/83
K170-42	10/17/83
K174-27	10/3/83
K189-11	10/31/83
K192-12	10/25/83
K193-5	10/26/83
K146-62	11/15/83
K157-58	11/8/83
K183-20	11/16/83
K158-102	12/13/83
K171-38	3/2/84
K188-19	4/24/84
K194-13	4/25/84
K195-17	5/30/84
K202-2	5/3/84
K196-19	6/27/84
K197-20	6/27/84
K204-6	7/6/84
K181-35	8/10/84
K194-19	9/12/84
K196-22	9/12/84
K156-91	12/20/84
K180-38	12/12/84
K184-37	12/10/84
K186-44	12/6/84
K194-25	12/14/84
K195-29	12/3/84
K203-11	12/17/84
K159-58	1/24/85
K176-50	1/31/85
K199-23	1/31/85
K205-15	1/30/85
K171-52	2/13/85
K172-69	2/6/85
K177-46	2/20/85
K202-13	2/26/85
K170-65	3/6/85

Table B: Strains from Spain

#	STRAIN	SEROTYPE
1	H1827	19
2	H3070	23
3	H4004	6
4	H4151	6
5	H4364	23
6	H5018	(23)
7	H5036	6
8	H5041	9
9	H5219	6
10	H5224	6
11	H5229	14
12	H5367	9
13	H8792	6
14	H9300	23
15	H9885	6
16	H9890	6
17	H10788	6
18	H11125	6
19	H11128	6
20	H1832	19
21	H2990	23
22	H3409	23
23	H3875	6
24	H4284	14
25	H4352	6
26	H4365	(6)
27	H4360	15
28	H5023	(19)
29	H9881	(6)
30	H9882	(6)
31	H9886	(23)
32	H9887	19
33	H11126	9
34	H5005	(6)
35	H5006	9
36	H5016	(19)
37	H5030	(19)
38	H3408	19
39	H6938	21
40	H2073	14
41	H3532	19
42	H4003	6
43	H4055	19
44	H4362	35
45	H4876	19
46	H9299	35
47	H9624	3
48	H9626	8 (15)
49	H9877	19
50	H10046	28
51	H10211	18
52	H10723	12
53	H10789	2
54	H11127	3

Table C: Strains from USA, South Africa and New Guinea

	Serotype	Isolation date	Source	Geographic site
SP-108	15 B	1939		
R6				CDC,[a] Atlanta
L7-0170	8	1986	Blood	Kotzebue, Alaska
L7-0172	14	1986	Blood	Kotzebue, Alaska
L7-1074	12 F	1987	Blood	Kotzebue, Alaska
L7-0175	8	1987	Blood	Kotzebue, Alaska
L7-0178	14	1986	Blood	Anchorage, Alaska
L7-0180	14	1986	Blood	Anchorage, Alaska
L7-0088	14	1984	Blood	Bethel, Alaska
L7-0092	14	1983	Blood	Bethel, Alaska
L7-0094	14	1983	Blood	Bethel, Alaska
L7-0101	23 F	1984	CSF[b] and blood	Bethel, Alaska
L7-0114	14	1982	Blood	Bethel, Alaska
SA-3		1979	Nasopharnyx	South Africa
SA-23	6	1979	Nasopharnyx	South Africa
L7-0171	12 F	1986	Blood	Kotzebue, Alaska
L7-0173	12 F	1987	Blood	Kotzebue, Alaska
L7-0176	8	1987	Blood	Kotzebue, Alaska
L7-0177	14	1986	Blood	Anchorage, Alaska
L7-0179	14	1986	Blood	Anchorage, Alaska
SP-148	36	1942		CDC, Atlanta
SP-99	11 A	1942		CDC, Atlanta
SP-96	10 A	1938		CDC, Atlanta
SP-95	9 V	1939		CDC, Atlanta
SA-45526				South Africa
L7-0085	10 A	1984	Blood	Bethel, Alaska
L7-0087	4	1982	Blood	Bethel, Alaska
L7-0090	14	1984	Blood	Bethel, Alaska
L7-0096	14	1983	Blood	Bethel, Alaska
L7-0102	7 F	1984	Blood	Bethel, Alaska
L7-0104	14	1983	Blood	Bethel, Alaska
L7-0106	14	1983	Blood	Bethel, Alaska
L7-0108	14	1983	Blood	Bethel, Alaska
L7-0099	33 F	1983	Blood	Bethel, Alaska
L7-0115	4	1983	Blood	Bethel, Alaska
SA-34034	4	1984	CSF	South Africa
L7-0113	4	1984	Blood	Bethel, Alaska
L7-0662	19 F	1985	Blood	Bethel, Alaska
L7-0110	19 A	1983	Blood	Bethel, Alaska
L7-0660	19 F	1985	Blood	Bethel, Alaska
L7-0661	6 B	1985	Blood	Bethel, Alaska
VA-Ben	9	1987	Blood	New York
L7-0091	19 A	1983	Blood	Bethel, Alaska
L7-0095	19	1984	Blood	Bethel, Alaska
L7-0097	19 A	1984	Midear	Bethel, Alaska
L7-0100	19 A	1983	Blood	Bethel, Alaska
L7-0103	18 C	1984	Blood	Bethel, Alaska
L7-0105	19 A	1984	Blood	Bethel, Alaska
L7-0107	19 A	1984	Blood	Bethel, Alaska
L7-0111	19 A	1983	Blood	Bethel, Alaska
L7-0112	6 B	1982	Blood	Bethel, Alaska
VA-Bri	15	1987	Blood	New York
L7-0086	23 F	1983	Blood	Bethel, Alaska
L7-0089	4	1985	Blood	Bethel, Alaska
L7-0109	14	1985	Blood	Bethel, Alaska
SA-45588	14	1985	Blood	South Africa
SA-13979	6	1985	Blood	South Africa
SA-42607	6		Pus	South Africa
SA-45443	19	1985	Blood	South Africa
P-3003	6			Papua, New Guinea
Glu	23	1987	Blood	Seattle
G-285	23	1987	Nasopharynx	New York
G-777	23	1987	Nasopharynx	South Africa
SA-34142	19	1984	Blood	South Africa
SA-23301	19	1985	Blood	South Africa
SA-381	19	1979	Blood	South Africa
SA-14750			Blood	

What is claimed is:

1. A method for preventing the expression of an undesired gene product in a pneumococcal strain without substantially altering the phenotype which comprises inserting a plasmid in the said strain so as to interrupt a gene for the said undesired gene product and growing the strain in an appropriate nutrient medium.

2. The method of claim 1 wherein the plasmid has a detectable marker.

3. The method of claim 2 wherein the strain desirably expresses pneumococcal polysaccharide, the undesired gene product, the expression of which is prevented, is lysin or autolysin, the plasmid comprises ermC, the interrupted gene comprises a lytA coding sequence, and the marker is resistance to erythromycin.

4. In a method for obtaining a desired product from the gene expression of a growing pneumococcal strain comprising growing said strain in a nutrient medium or culture broth and recovering the said desired product from said medium or broth and/or from said strain, the improvement comprises employing a strain which has had a plasmid inserted into a gene for an undesired gene product so as to interrupt a coding sequence for said undesired gene product without substantially altering the phenotype and to thereby prevent the expression of said undesired gene product.

5. In the method of claim 4, the plasmid comprises a plasmid having a detectable marker.

6. In the method of claim 5, the desired product comprises pneumococcal polysaccharide, the undesired gene product comprises lysin or autolysin, the plasmid comprises ermC, the coding sequence for the undesired gene product comprises a lytA coding sequence, and the marker is resistance to erythromycin.

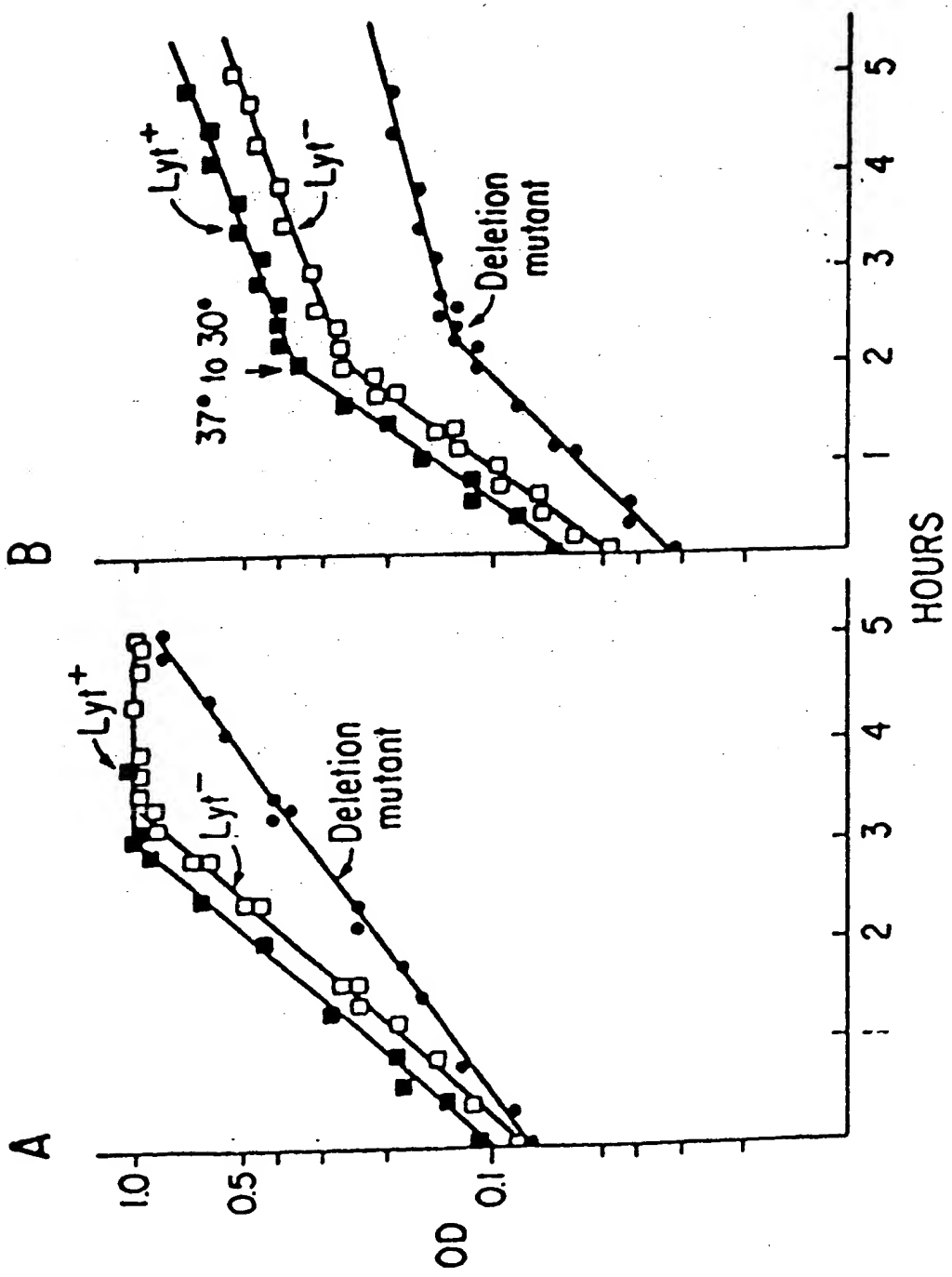

Fig. 1

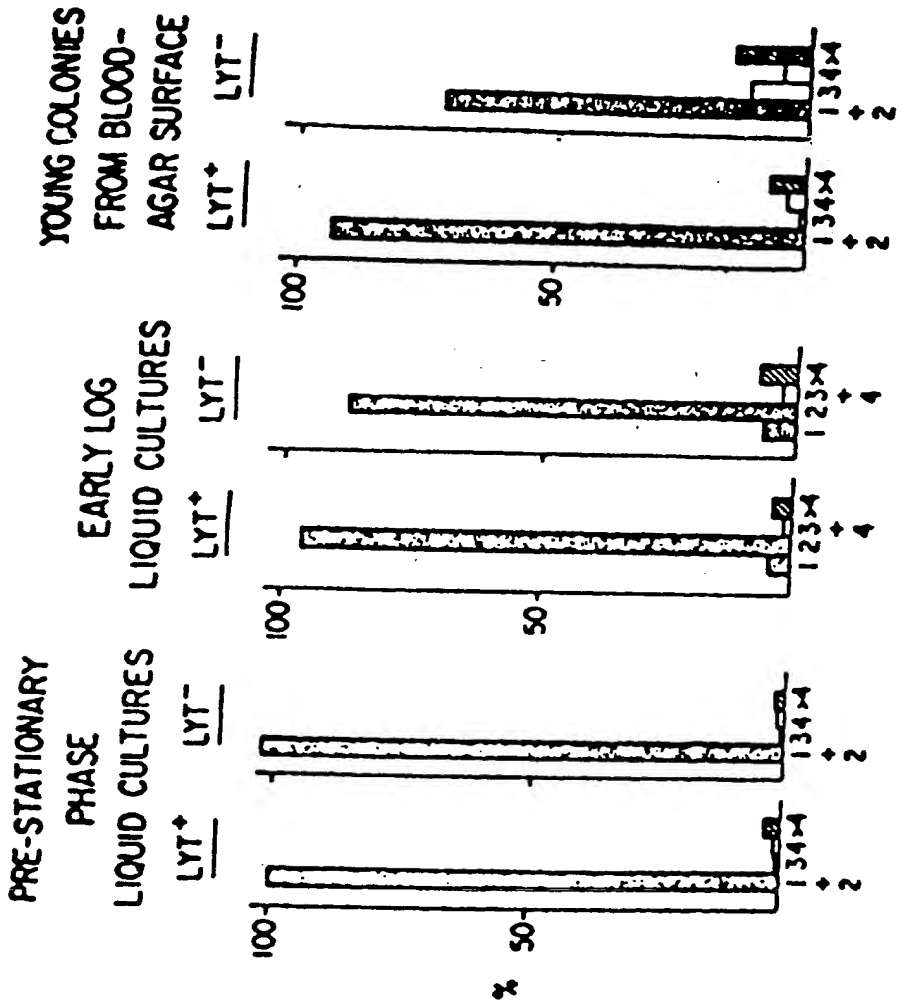

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/03258

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/06; C12N 15/00; C07H 1/06, 1/08, 5/04, 5/06

US CL: 435/69.1, 172.1, 172.3, 320.1; 536/55.1, 127

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S	435/69.1, 172.1, 172.2, 172.3, 320.1, 536/55.1, 127

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Databases: STN (CA)
Automated Patent System (File USPAT 1971-1990

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Infection and Immunity, vol. 57, No. 7, issued July 1989, Berry et al., "Reduced Virulence of a Defined Pneumolysin-Negative Mutant of Streptococcus pneumoniae". pages 2037-2042, see Fig. 2-3, page 2039 and Fig. 4, page 2040.	1-6
X	Journal of Bacteriology, vol. 170, No 12, issued December 1988, Tomasz et al, "Insertional Inactivation of the Major Autolysin Gene of Streptococcus pneumoniae", pages 5931-5934, see Fig. 1 and Fig 2, Table 1 and Table 2, and page 5933, column II, last paragraph.	1-6

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 August 1991	12 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Gian Wang

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATIO ONTINUED FROM THE SECOND SHEET

Y	Mol. Gen. Genet., vol. 204, issued 1986, Lopez et al., "Isolation, Characterization and Physiological Properties of an Autolytic -Defined Mutant of Streptococcus Pneumoniae". pages 237-242, see Fig 1-4 and page 239, column II.	1-6

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

. because they relate to parts of the international application that do not comply with the prescribed require- an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.